Mitochondrial MnSOD Expression in Ovarian Cancer: Role in Cell Proliferation and Response to Oxidative Stress

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Running Title: MnSOD in Ovarian Cancer

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Superoxide dismutases (SOD) are important antioxidant enzymes responsible for the elimination of superoxide radical (O2·-). The manganese-containing SOD (MnSOD) has been suggested to have tumor suppressor function and is located in the mitochondria where the majority of O2·- is generated during respiration. Although increased reactive oxygen species (ROS) in cancer cells has long been recognized, the expression of MnSOD in cancer and its role in cancer development remain elusive. The present study used human tissue microarray to analyze MnSOD expression in primary ovarian cancer tissues, benign ovarian lesions, and normal ovary epithelium. Significantly higher levels of MnSOD protein expression were detected in the malignant tissues compared to normal tissues (p<0.05). In experimental systems, suppression of MnSOD expression by siRNA caused a 70% increase of superoxide in ovarian cancer cells, leading to stimulation of cell proliferation in vitro and more aggressive tumor growth in vivo. Furthermore, stimulation of mitochondrial O2·- production induced an increase of MnSOD expression. Our findings suggest that the increase in MnSOD expression in ovarian cancer is a cellular response to intrinsic ROS stress, and that scavenging of superoxide by SOD may alleviate the ROS stress and thus reduce the simulating effect of ROS on cell growth.

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide (O2·-) and hydrogen peroxide (H2O2) are constantly produced during metabolic processes in all living species. Under normal physiological conditions, cellular ROS generation is counterbalanced by the action of antioxidant enzymes and other redox molecules. The balance between O2·- generation and elimination is important for maintaining proper cellular redox states. A moderate increase in ROS can stimulate cell growth and proliferation (1,2). However, excessive ROS accumulation will lead to cellular injury such as damage to DNA (3,4), protein (5) and lipid membrane (6). Because of their potential harmful effects, excessive ROS must be promptly eliminated from the cells by a variety of antioxidant defense mechanisms including important enzymes such as superoxide dismutase (SOD), catalase and various peroxidases. The cytosolic copper/zinc-containing SOD (Cu/ZnSOD, or SOD1) and the mitochondrial manganese-containing SOD (MnSOD, or SOD2) are two essential enzymes responsible for catalyzing the conversion of O2·- to H2O2, which is further eliminated by catalase and peroxidases (7). Since mitochondrial respiratory chain is a major site of O2·- generation in the cells, MnSOD plays an important role in maintaining cellular ROS balance.
Compelling evidence suggests that cancer cells are generally under ROS stress (8-10). Although the precise mechanisms responsible for increased ROS stress in cancer cells have not be defined, the increase in ROS generation is attributed to active cellular metabolic activity under the influence of oncogenic signals and/or to mitochondrial malfunction in cancer cells (11). ROS stress seems to render cancer cells more dependent on SOD to protect themselves from O$_2^-$-mediated damage (12). Indeed, previous studies showed increased levels of Cu/ZnSOD and MnSOD expression in cancer cells of various tissue origins (13-15). However, the relative expression levels of MnSOD in cancer cells and normal cells still remain controversial in the literature. For instance, increased MnSOD expression has been observed in experimental ovarian cancer animal model (16) as well as primary ovarian cancer tissues from patients (8,17). Analysis of blood samples from 30 ovarian cancer patients and an equal number of age-matched normal subjects showed significantly increased concentrations of plasma thiobarbituric acid reactive substances and conjugated dienes in the patient specimens, indicating increase oxidative stress in ovarian cancer (18). However, the same study also showed low levels of SOD, catalase, vitamin C and vitamin E in the plasma of the patient blood samples, possibly due to their increased utilization to scavenge lipid peroxides as well as their sequestration by tumor cells (18). This is in contrast with the increased serum MnSOD observed in another study (17). Decreased MnSOD activity and expression were also reported in certain colorectal carcinoma, and pancreatic cancer cells (19,20). These apparent conflicting observations are likely due to different assays and various cell types used in those studies. Thus, it is important to clarify this issue by further examining the expression levels of SOD in a large number of primary human cancer tissues in comparison with normal tissues. Tissue microarray analysis provides an effective tool for such analyses. This new technique was use in the present study to compare the expression of MnSOD and CuZnSOD in primary human ovarian cancer tissues, benign ovarian lesions, and normal tissues.

While the biochemical activity of MnSOD in catalyzing the conversion of O$_2^-$ to H$_2$O$_2$ in the mitochondria has been well characterized, the potential role of MnSOD in cancer development remains to be defined. Because MnSOD level seems decreased in certain cancer cells and forced expression of MnSOD appears to suppress malignant phenotypes in certain experimental model, this molecule has been considered to be a tumor suppressor (21). On the other hand, high levels of MnSOD expression have been detected in various primary human cancer tissues and in blood samples from patients with various types of leukemia (22,23). The increase in expression of MnSOD appears inconsistent with its proposed tumor suppression function. It is also known that SOD expression is inducible by oxidative stress, which has been demonstrated in several experimental systems (24-26). Thus, it seems reasonable to hypothesize that increased MnSOD expression may be a cellular response to intrinsic oxidative stress in cancer cells. The increased SOD activity decreases superoxide content in the cells and thus reduces the ROS-mediated stimulation of cell growth. If this were the case, MnSOD would decrease cancer cell proliferation indirectly through reduction of ROS, unlike conventional tumor suppressors, which usually regulate cells growth and show decreased expression in cancer tissues. One major goal of the present study was to test this hypothesis. We first used comparative tissue microarray analysis to answer the question of the relative levels of MnSOD protein in human ovarian carcinoma tissues compared with the normal epithelium and benign lesions. The role of MnSOD in determining ROS levels in ovarian cancer cells and in regulating cell proliferation and tumor growth was further evaluated both in vitro and in vivo. Our study showed that MnSOD level was significantly higher in malignant ovarian cancer tissues than the normal ovarian epithelium and benign lesions. Suppression of MnSOD expression caused accumulation of ROS, leading to increased cell proliferation in vitro and rapid tumor growth in vivo.

**EXPERIMENTAL PROCEDURES**

**Cell culture and analysis of cell proliferation.**

The ovarian cancer cell line SKOV3 was maintained in RPMI 1640 medium containing 10% fetal bovine serum. Colony formation
analysis was used to measure cell proliferation, the same numbers of SKOV3 cells transfected with either MnSOD-siRNA or the control U6 vector were plated in duplicate of 6-well plates. Ten days later, the colonies were fixed with a solution containing 50% methanol and 5% acetic acid for one hour at room temperature. The fixation solution was then aspirated off and the plates were allowed to dry. The cell colonies were stained with 1:20 dilution of Giemsa stain (Sigma diagnostics, St. Louis, MO) for one hour at room temperature. The stained colonies were scored and the percentages of colonies formed of each cell line were compared.

Ovarian Cancer Tissue Microarrays.

Ovarian tissue specimens from patients with primary epithelial ovarian cancer who had undergone initial surgery at The University of Texas M. D. Anderson Cancer Center between 1990 and 2001 were used for tissue microarray analysis with proper informed consent. Hematoxylin-eosin stained sections were reviewed by a pathologist to identify representative areas of the tumors from microarray analysis. Tissue microarray blocks were constructed by taking core samples from morphologically representative areas of paraffin-embedded tumor tissues and assembling them on a recipient paraffin block. This was accomplished by using a precision instrument (Beecher Instruments, Silver Spring, MD) with two separate core needles for punching the donor and recipient blocks and a micrometer-precise coordinate system for assembling tissue samples on a block. For each tissue specimen, two replicate samples (1-mm diameter) were collected and each was placed on a separate recipient block. Morphologically normal tissues and benign ovarian cystadenomas were also placed on the same tissue array for comparison. Each block contained 4 normal ovarian tissues, 9 serous benign cystadenomas, 8 serous and 11 mucinous low malignant potential tumors, 11 serous low-grade carcinomas and 20 serous high-grade carcinomas.

Analysis of MnSOD and CuZnSOD Expression in Tissues on Microarrays.

The tissue microarray slides were subjected to immunohistochemical staining. After initial deparaffinization, endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide. Deparaffinized sections were microwaved in 10 mM citrate buffer (pH 6.0) to unmask the immunoeptopes. The slides were incubated at 37°C for 2 hours with a 1:50 dilution of MnSOD antibody (Biodesign) and 1:200 of CuZnSOD antibody (Calbiochem), then with biotin-labeled rabbit anti-sheep secondary antibody (Calbiochem, 1:500) for 30 minutes, and finally with a 1:40 solution of streptavidin:peroxidase (Biocare, Walnut Creek, CA) for 20 minutes. The samples were then stained for 5 minutes with 0.05% 3',3'-diaminobenzidine tetrahydrochloride that had been freshly prepared in 50 mM Tris buffer at pH 7.6 containing 0.024% H2O2 and then counterstained with hematoxylin, dehydrated, and mounted. All antibody dilutions and streptavidin-peroxidase were in phosphate-buffered saline (pH 7.4) containing 1% bovine serum albumin. Negative controls were made by replacing the primary antibody with phosphate-buffered saline to ensure no false-positive staining. The immunostained tissue microarrays were scored using computerized digital analysis system (Ariol SL-50, Applied imaging, California). Counting criteria and software settings were identical for all slides. Total cytoplasmic stained area was expressed in pixels. Quantitation was done blinded to pathology information. Normal ovarian epithelial cells were used as a comparison for intensity and pattern of staining. The mean value of two replicate tissue cores from each tumor specimen was considered for each case. Total integrated optic density was expressed in arbitrary optic density units, which were then grouped as low (weak SOD signal intensity similar to the normal ovarian control with less than 25% variation), intermediate (25-50% increase over control), or high (more than 50% increase over control) levels. Statistical differences between tissue groups were evaluated by Mann-Whitney U test and Kruskall-Wallis ANOVA test as appropriate. Results were considered statistically significant at the p< 0.05 level.

MnSOD-siRNA vector and transfection.

DNA oligonucleotides encoding siRNA with loop sequence CATTGG were subcloned into the pBabe/U6-puromycin' promoter vector (a gift from Dr. Y. Shi of Harvard University) as described previously (27). The siRNA sequence targeting MnSOD was selected from Genbank (Accession
No. NM_000636). After homology analyses by nucleotide sequence BLAST, we chose the coding region of MnSOD mRNA between 114-134 nucleotide (5’-GGAACCUCACAUCAACGCGCA-3’) as the siRNA target. The constructed MnSOD-siRNA vector was verified by DNA sequencing analysis. To create amphotropic retrovirus, the phoenix cells were subjected to calcium-mediated transfection with 20–25 µg of the control pBabe/U6 vector or pBabe/U6/MnSOD-siRNA. The RPMI 1640 medium (containing 10% FBS, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 µg/ml streptomycin) was changed 9–12 h after transfection, and the cells were incubated for another 12–14 hours at 37°C, after which the plates were moved to a 32°C incubator for 48 hours to increase the viral titer. Retroviruses were harvested using the previously described method (28), and used for infecting the human ovarian cancer cells SKOV3 at 40% confluence in medium containing 4µg/ml polybrene (Sigma). After infection (24 h), the cells were selected in the medium containing puromycin (1 µg/ml). The surviving colonies were pooled and cultured in the medium without puromycin. Inhibition of MnSOD expression was verified by western blot analysis, which showed that stable suppression of MnSOD were maintained for more than one year without further selection pressure (puromycin).

Analysis of cellular MnSOD, CuZnSOD, and Superoxide.

Western blot was performed to determine the protein expression of MnSOD, CuZnSOD, and β-actin in SKOV3 cells and the transfectants. The same amount of protein lysates were loaded on a 12% SDS-PAGE gel, followed by immunoblot analysis using anti-CuZnSOD (Calbiochem), anti-MnSOD (Biodesign), and anti-β-actin (Sigma) as described previously (8). Cellular superoxide was measured by flow cytometry using a fluorescent dye hydroethidine (Molecular Probes) as described previously (8). After incubation with hydroethidine (100 ng/ml) at 37°C for 1 h, the samples were washed with PBS twice, and analyzed by a Becton Dickinson FACSCalibur flow cytometer (Moutain View, CA). The data were analyzed using the Becton Dickinson CellQuest Pro software package.

RESULTS

Human tissue microarray analysis was first used to evaluate the MnSOD and CuZnSOD expression in ovarian cancers in comparison with benign ovarian lesions and normal ovary epithelium. Primary tissue slides containing tissue punches of normal ovary epithelium, benign cystadenomas, low malignant potential (LMP) lesions, low grade and high-grade ovarian carcinomas were immunostained for MnSOD and CuZnSOD protein expression, and the intensity of the SOD signal was analyzed as described in Materials and Methods. As illustrated in Figure 1, both normal tissue and benign lesions exhibited only a weak MnSOD signal and CuZnSOD(Fig. 1A-1B, 1F-1G). In contrast, the malignant lesions showed substantially higher levels of MnSOD and CuZnSOD staining (Fig. 1C-1E, 1H-1J). This increase in SOD signal in cancer tissues was not due to an uneven immunostaining, since the stromal cells and the connective tissues within the cancer tissues also showed weak SOD staining, comparable to that observed in normal ovarian tissue. The increase in SOD protein expression in ovarian cancer cells was further confirmed by western blot analysis of protein extracts from freshly frozen ovarian serous cancer tissues, benign serous ovarian cystadenomas, and normal ovary tissues. As shown in Fig. 2A, the protein extracts from the majority of ovarian carcinoma tissues contained higher levels of MnSOD and CuZnSOD than the normal or benign tissues.

Further analysis of 62 samples on the tissue microarray revealed an increase in MnSOD expression in malignant tumors compared to normal ovarian surface epithelial cells and benign tumors (p < 0.001, Kruskal-Wallis test). Consistently, low levels of MnSOD expression were found in normal ovarian surface epithelial cells and benign tumors (Fig. 2B). In contrast, the majority of the malignant tumors exhibited either intermediate or high levels of increase in MnSOD expression, albeit some variability among the individual samples. Using the same statistical analysis, we also found similar increase of CuZnSOD expression in malignant tumors compared to the benign lesions and normal tissues (Fig. 2C). These data together suggest a possibility that the increased SOD expression may reflect the
cellular response to intrinsic oxidative stress in the cancer cells.

The increased MnSOD expression in primary ovarian cancer tissues prompted us to further investigate the potential role of this molecule in regulating ovarian cancer cell proliferation in vitro and tumor growth in vivo. We first used small interference (siRNA) technique to specifically knock down the expression of MnSOD in human ovarian cancer cells (SKOV3), and then examined possible alterations in cellular ROS contents and growth behavior. As illustrated in Fig. 3A, the MnSOD siRNA target sequence was cloned into the pBabe/U6-\textit{puromycin}\textsuperscript{r} vector for stable expression in SKOV3 cells, while the empty pBabe/U6-\textit{puromycin}\textsuperscript{r} was used as the vector control. The stably transfected cell clones were pooled and protein extracts were analyzed for expression of MnSOD, Cu/ZnSOD, and β-actin. As shown in Fig. 3B, the MnSOD siRNA specifically suppressed the expression of MnSOD (lane 3) and did not affect the expression of Cu/ZnSOD or β-actin. The control vector pU6 exhibited no effect on the expression of all these molecules tested.

Since MnSOD catalyzes the elimination of O\textsubscript{2} in the mitochondria where a large portion of cellular ROS is generated, we measured the O\textsubscript{2} content in SKOV3 cells before and after the suppression of MnSOD expression by siRNA. As shown in Fig. 3C, flow cytometric analysis revealed that there was a 70% increase in O\textsubscript{2} content in cells transfected with MnSOD siRNA, whereas the empty vector pU6 did not cause any significant change in cellular O\textsubscript{2} level. These data suggest that the knockdown of MnSOD expression alone without suppressing Cu/ZnSOD was sufficient to cause an accumulation of O\textsubscript{2} in the cells.

It is known that a moderate increase in ROS can stimulate cell proliferation and contribute to cancer development (29,30). We used several assays to test the possible effect of MnSOD suppression and the subsequent O\textsubscript{2} accumulation on cancer cell proliferation in tissue culture and tumor growth in an experimental animal model. Direct measurement of cell counts showed that the siRNA-transfected cells consistently outgrew the control vector-transfected cells. This was further confirmed by a colony formation assay (Fig. 4A and 4B, \textit{p}<0.01). Ten days after the same numbers of cells transfected either MnSOD-siRNA or the empty vector were seeded, the MnSOD siRNA-transfected cells formed larger colonies than the vector control cells, although the plating efficiency (number of colonies) for both cells appeared similar.

Animal experiments were performed to test if suppression of MnSOD can promote the growth of ovarian cancer cells in vivo. The siRNA-transfected SKOV3 cells were inoculated subcutaneously on the left flanks of 20 nude mice (2x10\textsuperscript{6/mouse}), and the same number of control SKOV3 cells transfected with pU6 vector were inoculated on the right flanks of the same mice for comparison. The ovarian cancer cells with suppressed MnSOD expression exhibited a significant growth advantage over the control cells transfected with the empty vector, as evidenced by the substantially larger tumor mass on the left flanks of the mice (Fig. 4C, D). Statistical analysis of data pairs showed a \textit{p} value of less than 0.05, indicating a significant difference between the two tumor growth curves. This is consistent with the observations of cell growth in vitro (Fig. 4A-4B). Taking together, these data suggest that suppression of MnSOD expression and the subsequent increase in cellular O\textsubscript{2} promote cancer growth both in vitro and in vivo.

To further test the possibility that the increased MnSOD expression in ovarian cancer cells may be induced by ROS stress, we use a biochemical method to enhance the generation of O\textsubscript{2} in mitochondria by incubating SKOV3 cells with a low concentration (0.1 µM) of rotenone, a mitochondrial respiratory complex I inhibitor known to promote electron leakage from the respiratory chain and increase ROS generation (31). O\textsubscript{2} contents and expression of MnSOD were measured at various times after rotenone incubation. As shown in Fig. 5, rotenone caused a significant increase in O\textsubscript{2} generation and substantial induction of MnSOD expression in SKOV3 cells. The increase in O\textsubscript{2} content and MnSOD expression was detected as early as 6 hour after rotenone incubation and lasted for at least 24 h. Both O\textsubscript{2} and MnSOD eventually decreased. These results suggest that cells were able to rapidly respond to ROS stress and enhance the expression of MnSOD. Thus, the increased MnSOD protein expression observed in primary ovarian cancer tissues may reflect the cellular...
response to intrinsic oxidative stress in the malignant cells.

DISCUSSION

MnSOD and CuZnSOD are major antioxidant enzymes that play important roles in scavenging superoxide radical and thus protect cells from free radical-mediated damage (7). Although increased oxidative stress and aberrant SOD expression in cancer cells have long been recognized (32), there have been conflicting reports on the relative levels of SOD expression in cancer cells compared to their normal counterparts. Increase of SOD has been detected in different types of cancer cells using various techniques (33-35). Other studies, however, showed that SOD levels or activities in cancer tissues appeared unchanged or even lower compared to normal tissues (19,36). Thus, it is important to analyze a large number of primary cancer tissues and normal samples under comparable assay conditions so that more conclusive data can be obtained. Tissue microarray analysis provides an effective tool to evaluate protein expression in a large number of primary tissues under the same assay conditions. In the present study, we first used this method to compare MnSOD and CuZnSOD expression in human primary ovarian cancer tissues, benign ovarian cystadenoma, and normal ovary epithelium. The results demonstrated that the majority of malignant ovarian tissues express significantly higher levels of both SOD than normal cells or tissues with benign lesions. This increase in SOD expression in cancer tissues was further confirmed by western blot analysis. Our data support the conclusion that SOD expression levels are increase in primary human cancer tissues.

It should be noted, however, that the expression of SOD is heterogeneous among the individual ovarian cancer samples (Fig. 2B). Among 49 malignant tissue samples, 15 samples (approximately 30%) showed a low level of MnSOD expression, whereas 34 samples (approximately 70%) exhibited increased MnSOD. This was significantly different from the uniformly low MnSOD expression in the normal and benign ovarian tissues. Similarly, the majority (41/49, or approximately 84%) of ovarian cancer tissues exhibited high levels of CuZnSOD expression, although there was also some heterogeneity among the individual samples (Fig. 2C) Nevertheless, the individual variation among cancer samples may be a possible reason for the conflicting reports on the relative levels of SOD expression in cancers. These observations also suggest that caution should be exercised in drawing conclusion from studies using a small number of primary cancer tissues.

The precise reason why the majority (70-80%) of ovarian cancer tissues expressed increased levels of SOD remains unclear at the present time. Growing evidence suggests that cancer cells produce high levels of ROS and are constantly under oxidative stress (8-10). Such intrinsic ROS stress may be a primary biochemical event that induces increased SOD expression. Since the expression of SOD is known to be responsive to oxidative stress ((24-26), increased SOD expression is generally attributed to enhanced ROS generation in cancer cells (11). Indeed, our study demonstrated that increasing superoxide generation by pharmacological interference of the mitochondrial respiratory chain by rotenone, which causes electron leakage and produces superoxide (31), induced a rapid increase of MnSOD expression in cultured ovarian cancer cells. Thus, the relatively higher level of MnSOD in cancer cells is likely secondary to the buildup of oxidative stress in the malignant tissues. Although the precise mechanisms responsible for the intrinsic oxidative stress in cancer remain to be defined, several potential mechanisms have been suggested. Oncogenic signals such as c-myc, Ras, and Bcr-Abl have been shown to cause increased ROS generation (11,37). Mitochondrial mutations and respiratory malfunction may also lead to increased production of superoxide (11). MnSOD expression has been shown to be inducible by multiple factors such as hypoxia, ROS, and inflammatory cytokines including IL-1 and IL-6 (38,39). It would be interesting in future studies to measure the expression of cytokines such as IL-1 and IL-6 in primary cancer tissues. It is possible that these inflammatory cytokines may function as the mediators of ROS-induced increase of MnSOD expression. In addition, the promoter of MnSOD contains binding sites for several transcriptional factors such as AP-1 and NFκB (40). ROS might also induce the upregulation of MnSOD by modulating the redox states of these transcriptional factors.
factors. The molecular mechanisms by which the expression of CuZnSOD responds to ROS stress have not been well characterized.

The increase of MnSOD expression in cancer cells may not be viewed as merely a cellular adaptation to cope with ROS stress. MnSOD may play an important role in regulating cell proliferation and tumor growth through its ability to regulate the level of cellular O$_2^-$, a free radical known to be involved in signaling cell growth and proliferation (29,30). A moderate increase of ROS such as O$_2$ or H$_2$O$_2$ is able to stimulate cell cycle progression and promote cell proliferation and survival. These signaling processes are thought to be achieved through redox modification of signaling molecules such as protein kinases and transcription factors, including MAPK, SAPK, JNK, NFkB, HSF1 and p53 (41). However, it is unclear which ROS species (superoxide or hydrogen peroxide) is mainly responsible for stimulating cell proliferation. Because there are active inter-conversions among ROS species in the biological system and an alteration in one ROS species often leads to change in other ROS species in whole cells, clear identification of a specific ROS species responsible for promoting cell proliferation. Because there are active inter-conversions among ROS species in the biological system and an alteration in one ROS species often leads to change in other ROS species in whole cells, clear identification of a specific ROS species responsible for promoting cell growth is a challenging task. Because suppression of MnSOD expression by siRNA leads to increase in O$_2^-$ and decrease in H$_2$O$_2$, it is likely that O$_2^-$ is the main ROS species that stimulates cell growth in our experimental system. Nevertheless, the increased expression of MnSOD would minimize the buildup of cellular ROS, and thus reduce its stimulation on cell proliferation. The decrease of oxidative stress subsequent to increased SOD expression could also reduce the risk of ROS-mediated DNA damage and genetic instability. As such, MnSOD has been considered by some authors as a tumor suppressor molecule. Indeed, previous studies showed that introduction of exogenous MnSOD to the transformed cell lines suppressed the malignant cell behaviors (21). Interestingly, over expression of MnSOD in SKOV-3 cells has been shown to suppress cell growth with concomitant increase in H$_2$O$_2$ and alteration of activation of p38MAPK activity (42).

Unlike the classical tumor suppressors such as p53 and PTEN, which are often mutated, deleted or unexpressed in cancer cells, functional MnSOD protein is highly expressed in cancer tissues in response to oxidative stress. Thus MnSOD is different from typical tumor suppressors in that it reduces cancer cell growth indirectly through elimination of superoxide, and that it is highly expressed in cancer tissues.

The high expression of MnSOD in certain primary human cancer tissues has led some investigators to suggest using MnSOD as an indicator to monitor cancer development and progression (43). However, it appears contradictory that MnSOD functions as a tumor suppressor and also serves as a cancer marker. The unique biochemical function of MnSOD and its upregulation by ROS stress provide a mechanistic explanation for this apparent discrepancy. The expression of MnSOD and its ability to rapidly respond to ROS stress provide a mechanism for the cells to maintain a proper level of superoxide. The loss of such antioxidant mechanism would lead to accumulation of superoxide and stimulation cell proliferation and tumor growth. This was demonstrated in our study using siRNA to suppress MnSOD expression (Fig 4). It is interesting to note that in certain types of cancer cells SOD levels appeared decreased (19,20), which might lead to O$_2^-$ accumulation and enhanced cell proliferation. However, in a majority of the primary ovarian cancer tissues the mechanism that upregulates MnSOD expression in response to ROS stress appeared to be intact, rendering increased MnSOD protein expression. The individual variations in MnSOD expression among different cancer tissues likely reflect the various degrees of ROS stress and other yet unknown changes in the respective cancer tissues. Despite this variation, the overall MnSOD expression in cancer tissues is significantly higher than in normal and benign tissues.

It is interesting to note that specific suppression of MnSOD by siRNA without affecting CuZnSOD expression cause a 70% increase of cellular O$_2^-$. This observation suggests that MnSOD is very important in regulating the overall balance level of O$_2^-$. Since MnSOD is mainly located in the mitochondria, the increased O$_2^-$ in the siRNA-transfected cells was most likely generated in the mitochondria. Importantly, this level of O$_2^-$ increase (70%) did not cause severe damage to the cells. Instead, it stimulated cell proliferation in vitro and promoted tumor growth in vivo. It is possible that the normal expression of
CuZnSOD in the cytosol may prevent $O_2^-$ from reaching the nucleus and damaging nuclear DNA, and thus reduce the risk of severe damage to the cells. The accumulated $O_2^-$ may stimulate cell growth by affecting the redox states of important cellular molecules such as transcriptional factors and cell cycle regulatory proteins (41,44).

In summary, our study demonstrated that there was a significant increase of SOD expression in ovarian cancer tissues. In view of conflicting reports on SOD expression levels in cancer cells, our study is significant in that the tissue microarray analysis provide strong evidence for increased SOD expression in primary human ovarian cancer tissue in direct comparison with normal or benign ovary tissues. We also demonstrated in cultured ovarian cancer cells that increase ROS stress was able to induce MnSOD expression, suggesting that the increase of SOD expression observed in primary patient samples was likely a cellular response to intrinsic oxidative stress in the cancer tissues. Another significant finding is that suppression of MnSOD expression by siRNA caused an accumulation of $O_2^-$ and promoted cell proliferation in vitro and tumor growth in vivo. Thus, in cancer cells the increase of MnSOD expression may prevent further increase of $O_2^-$ and thus reduce its stimulatory effect on cell proliferation. As such, MnSOD may be considered as a protective molecule capable of counteracting the harmful effect of ROS including stimulation of cells growth and causing DNA damage. Unlike classical tumor suppressor genes, MnSOD expression is increased in cancer tissues due to upregulation in response to oxidative stress in cancer cells.

**REFERENCE**


**FOOTNOTE**
This study was supported in part by Grants CA85563, CA100428, and CA109041 (P.H.) from the National Cancer Institute, the National Institutes of Health, and RSG-04-028-1-CCE (J.L.) from the American Cancer Society.
The abbreviations used are: O$_2^-$, superoxide radical; ROS, reactive oxygen species; SOD2 or MnSOD, manganese-containing SOD; SOD1 or Cu/ZnSOD, copper/zinc-containing SOD.

**FIGURE LEGENDS**

**Fig. 1.** MnSOD and CuZnSOD protein expression in normal ovarian epithelium, benign ovarian cystadenomas, and ovarian carcinomas. Immunostaining of human ovarian cancer tissue microarrays is described in Materials and Methods. Representative tissue punches are shown. (A-E), Immunostaining of anti-MnSOD. (F-J), Immunostaining of anti-CuZnSOD. A and F, Normal ovarian epithelium and stromal tissue. B and G, Benign serous cystadenoma. C and H, Serous low-malignant-potential (LMP) tumor. D and I, Low grade serous carcinoma. E and J, High grade serous carcinoma.

**Fig. 2.** Increased SOD expression in human ovarian cancer tissues. A, Western blot analysis of MnSOD and CuZnSOD in protein extracts from normal ovary tissue, benign ovarian cystadenoma, and ovarian carcinoma. β-actin was also blotted as a control for protein loading. Lanes 1-2, protein extracts of normal ovarian tissues; lanes 3-5, benign serous ovarian cystadenoma; lanes 6-12, high grade serous ovarian carcinoma. B-C, Comparison of MnSOD (B) and CuZnSOD (C) expression in normal ovary tissue, benign ovarian cystadenoma, and ovarian carcinoma. MnSOD expression was analyzed by tissue microarray analysis as described in Materials and Methods. S-LMP, serous low-malignant-potential. M-LMP, mucinous low malignant potential. NS, not significant. Both normal epithelium and benign lesions exhibited low MnSOD expression with no statistical difference. The p values represent the levels of significance comparing MnSOD expression between the indicated cancer tissues and normal/benign tissues using the Mann-Whitney U Test. The numbers in parenthesis indicate the numbers of tissue specimens (n).

**Fig. 3.** Suppression of MnSOD expression and accumulation of superoxide by siRNA. A, Vector map of the MnSOD-siRNA construct used in this study. The DNA sequence coding for the hairpin siRNA targeting MnSOD was subcloned into the pBabe-puromycin$^+$ retroviral vector under the control of the U6 promoter. See Materials & Methods for detail. B, Expression of MnSOD and CuZnSOD in the control SKOV3 cells and cells stably transfected with the MnSOD-siRNA vector or the U6 control vector. Protein lysates from the indicated cells were immunoblotted with specific antibodies against MnSOD, CuZnSOD, and β-actin. Lane 1, control SKOV3 cells; lane 2, cells stably transfected the U6 control vector; lane 3, cells stably transfected with the MnSOD-siRNA vector. C, Flow cytometric analysis of O$_2^-$ content in the control SKOV3 cells and cells stably transfected with the MnSOD-siRNA vector or the U6 control vector. Cells were labeled with hydroethidine (100 ng/ml) for 60 min followed by flow cytometric analysis as described in Materials and Methods.

**Fig. 4.** Comparison of cell proliferation in SKOV3 cells with different MnSOD expression levels. A, Same number of cells (700/well) stably transfected with either MnSOD-siRNA vector or the U6 control vector were seeded in a 6-well plate and incubated for 12 days. Cell colonies were fixed, stained and photographed. B, Quantitative comparison of colony formation in SKOV3 cells stably transfected with either MnSOD-siRNA or U6 control vector. Data are expressed as the means and standard deviations of six determinations (two separate experiments, three wells each). (*) indicates p<0.01 using the Student t test. C, SKOV3 cells stably transfected with MnSOD-siRNA vector were inoculated subcutaneously on the left flank of 20 mice (2 million cells/mouse), while the same number of control cells transfected with the U6 empty vector were inoculated on the right flank of the same mice. Tumor sizes were measured at the indicated times and expressed as the mean volume ± SE. Two tailed t test of all the paired data points showed a p value of 0.0444, indicating a significant difference between the two tumor growth curves. A representative mouse bearing tumors on both flanks (day 30) is shown on the top panel and the quantitative data shown in the lower panel.
**Fig. 5.** Induction of increased MnSOD expression by rotenone-mediated ROS accumulation. **A,** Increased superoxide generation in SKOV3 cells treated with rotenone. Cells were incubated with a low concentration (100 nM) of rotenone for 6 or 36 h, and cellular O$_2^-$ levels were measured by flow cytometric analysis. **B,** Western blot analysis of MnSOD expression in SKOV3 cells treated with rotenone. Cells were incubated with 100 nM rotenone for the indicated time, and cell lysates were analyzed for expression of MnSOD by immunoblotting. β-actin was also blotted as a control for protein loading.
Figure 1

MnSOD

A
Normal

B
Benign

C
LMP

D
Low Grade

E
High Grade

CuZnSOD

F

G

H

I

J
Figure 2

A

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B

C

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Figure 3

A

pBabe/U6/MnSOD/siRNA

siRNA transcript product:

GGGAACCUCUCAUCACCGCA C^A U
3'-UUUUUCCUUGAGAGUGUUGCGCGU-

B

MnSOD

Cu/ZnSOD

β-Actin

C

SKOV3 MnSOD-siRNA

U6 vector

Cellular O_2^- content
Figure 4

A

U6 vector control MnSOD siRNA

B

Colony Formation, %

U6 Vector MnSOD:siRNA

C

MnSOD siRNA U6 vector control

Tumor vol, mm³

Time (days)

P = 0.044
Figure 5

A

B

Time (h): 0 6 12 24 36
MnSOD

β-actin
Mitochondrial MnSOD expression in ovarian cancer: Role in cell proliferation and response to oxidative stress
Yumin Hu, Daniel G. Rosen, Yan Zhou, Li Feng, Gong Yang, Jinsong Liu and Peng Huang

J. Biol. Chem. published online September 22, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M503296200

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